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(54) Title: cDNA PROBE FOR BREAST CANCER DIAGNOSIS AND TREATMENT**(57) Abstract**

Some cDNA probes cloned from an mRNA coded for by an isolated gene (designated Brush-1) located at 13q12-q13 that is useful in diagnosis and treatment of breast cancer are disclosed. The probe provides a means for detection of premalignant mammary cells and early detection of breast cancer. It is also useful in designing therapeutic treatments for these conditions by traditional pharmaceutical methods or gene therapy.

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CDNA PROBE FOR BREAST CANCER DIAGNOSIS AND TREATMENT

FIELD OF THE INVENTION

The invention herein was made with government support under contract 5P01 CA 44768-09 with the National Cancer Institute. The federal government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

Breast cancer is one of the most common malignancies found among women above the age of 35 and results in thousands of deaths in the United States each year. Current treatments, including radiation, chemotherapy and mastectomy, have been successful in halting or slowing the disease's progress, but these known treatments have many undesirable side effects of their own.

Early detection of the disease is essential to a positive prognosis for breast cancer treatment. Frequently, by the time a tumor is detected, the disease has already progressed to the point where successful treatment by known methods is difficult or impossible.

Research has produced some new compounds and methods for diagnosis and treatment of certain

types of cancerous conditions. U.S. Patent No. 5,262,528, for instance, is directed to a cDNA probe differentiating normal and cancerous tissues and U.S. Patent No. 4,942,123 is directed to a method of
5 diagnosis of retinoblastoma and "involved cancers", said to include breast cancer.

Inactivation of tumor suppressor genes may play an important role in human cancers, including breast cancer. See Ponder, B., *Nature (Lond.)* 335: 400-402 (1988); Sager, R., *Science* 246: 1406-1412
10 (1989). This is thought to occur by the inactivation of one allele and the subsequent loss or replacement of the other allele contained on a chromosomal segment. Several localized regions have been
15 implicated by the coincidence of their loss in various breast tumors. These include the short arms of chromosomes 3, 17 and 18 and sites on the long arms of chromosomes 1, 13 and 22. See Sato, T., et al., *Cancer Res.* 50: 7184-7189 (1990); Devilee, p.,
20 et al., *Int. J. Cancer* 47: 817-821 (1991); Chen, L-C, et al., *J. Natl. Cancer Inst.* 84: 506-510 (1992). Of particular interest is the chromosome 13q region which shows relatively frequent loss of heterozygosity (LOH) in breast tumors suggesting an
25 important role in breast cancer initiation and/or progression. See Lundberg, C., et al., *Proc. Natl. Acad. Sci. USA* 84: 2372-2376 (1987); Devilee, p., et al., *Genomics* 5: 554-560 (1989). Most of these studies have focussed on the q14 region which
30 contains the retinoblastoma (RB1) gene located at 13q14.2. RB1, the gene involved in heredity and sporadic retinoblastoma, was also the first gene identified and characterized as a tumor suppressor gene. See Stanbridge, E.J., *Functional evidence for*
35 *human tumour suppressor genes: chromosome and molecular genetic studies*, in GENETIC SURVEYS 12:

- 3 -

TUMOUR SUPPRESSOR GENES, THE CELL CYCLE AND CANCER 5-24 (1992)). The relationship between RB1 and breast cancer, however, is not clear. Subsequent studies have shown that the LOH for RB1 in breast cancer is not correlated with the loss of RB1 gene expression. See Borg, A., et al., *Cancer Res.* 52: 2991-2994 (1992). In addition, LOH in the region next to RB1 has been found in human breast carcinoma while the RB1 gene itself did not show such a genetic change. See Devilee, p., et al., *Genomics* 5: 554-560 (1989). Hence, upon closer re-examination of this region, the finding has been made that RB1 expression, at least for mRNA, apparently is not affected by LOH in the region which includes RB1. Instead, a proximal gene demonstrates the expected pattern of a tumor suppressor gene for breast cancer.

The gene cloned and sequenced as described herein, *Brush-1*, may represent yet another member of a new class of tumor suppressor genes that function directly as RNA or as the RNA component of a ribonucleoprotein as has been described for the H19 gene (Brannan et al., 1990, *Mol. Cell. Biol.* 10:28; Hao et al., 1993, *Nature* 365:764). Both *Brush-1* and H19 are expressed as a polyadenylated RNA; are expressed at higher levels in fetal as compared to adult tissues; contain multiple small open reading frames; are both conserved in the monkey genome (shown by zoo blot hybridization); are located in regions of frequent LOH; and show loss of RNA expression in tumors demonstrating this LOH.

Known applications of sequenced genes include use of the sequences or of RNA or amino acid sequences derived therefrom for diagnosis or treatment of the corresponding disease. Accordingly, it is useful for the diagnosis and treatment of

- 4 -

breast cancer to isolate and further characterize this gene in the region next to RB1.

An object of the present invention is to provide a cDNA probe derived from this gene
5 (designated Brush-1) useful in diagnosis and treatment of breast cancer.

Additional objects and advantages of the invention will be set forth in the description of the preferred embodiments which follows, and in part will
10 be obvious from the description, or will be learned by practice of the invention.

SUMMARY OF THE INVENTION

The present invention is directed to a novel DNA sequence complementary to an mRNA coded for
15 by an isolated gene (designated Brush-1) located at 13q12-q13 that is useful as a probe in diagnosis and treatment of breast cancer. The probe provides a means for detection of premalignant mammary cells and early detection of breast cancer. It is also useful
20 in designing therapeutic treatments for these conditions by traditional pharmaceutical methods or gene therapy.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings are incorporated
25 in and constitute a part of the specification.

Figure 1 depicts an autoradiogram of a Northern blot analysis of Brush-1 mRNA. 10 µg per

lane of polyadenylated RNA were analyzed by probing with the 1.5 kb Brush-1 cDNA representing the most 3'-region.

Figure 2 depicts an autoradiogram of a RT-PCR Analysis. Products from RT-PCR run on 1% agarose gel and stained with ethidium bromide. RNA source: lanes 2-4, CAMA1; 5-7, DU4475; 8-10, G94; 11-13, MDA468; Amplimers: lanes 2, 5, 8 & 11, Brush-1; lanes 3, 6, 9, 12, RBI; lanes 4, 7, 10, 13, β -Actin; pGEM marker: lanes 1 and 14.

Figure 3 depicts the position of Brush-1 cDNA clones relative to the 4.7kb mRNA.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The following describes the characterization of the gene (designated Brush-1) localized to the 13q region proximal to RB1 that is differentially expressed in normal versus tumor mammary epithelial cells and the isolation and derivation of mRNA and cDNA sequences therefrom. This description does not limit the invention and those of ordinary skill in the art will recognize that many variations on this method can be used with equivalent efficacy produce a cDNA probe.

The growth conditions for the various breast cancer cell lines and normal cells isolated from reduction mammoplasties are described in Smith, H.S., *In vitro models in human breast cancer in BREAST DISEASES, 2ND EDITION* 181-189 (J.R. Harris, et al., eds., 1991). Human breast primary tumor samples

- 6 -

and paired normal skin tissues were collected from 76 individuals. Tumor samples were dissected to remove most of the normal tissue, and stored in liquid nitrogen until use. If the skin tissues are too small to isolate sufficient DNA for analysis, cultured skin fibroblasts from the same patient may be used to extract DNA.

DNA isolation was performed as described by Kallioniemi, A., et al., *Cytogenet. Cell Genet.* 60: 190-193 (1992), incorporated by reference herein. Total RNA was isolated from both tissue culture cells and primary tumors using the Ultraspec[™] RNA method (Biotecx Laboratories, Inc., TX). Final RNA pellets were resuspended in DEPC water and then stored at -70°C. Procedures for polyadenylated mRNA selection and subsequent Northern analysis are described in Sambrook, J., Fritsch, E.F. & Maniatis, T., *MOLECULAR CLONING: A LABORATORY MANUAL* (1992).

Single stranded cDNA was synthesized by oligo(dT) priming (0.5 µg) from 3µg of total RNA using 20U of M-MLV Reverse Transcriptase (RT) (Gibco, BRL) in a final volume of 20 µL. The RT enzyme was inactivated by incubation at 70°C for 10 minutes and the product was diluted to 200 µl. A 5µl aliquot of cDNA was used directly for each PCR amplification. Specific amplification for each of three different mRNA species was achieved using sequence specific primers. Amplification primers for the β-Actin gene were obtained from Clontech Laboratories (Palo Alto, CA) and consisted of the following sequences; 5'-ATGGATGATGATATCGCCGCG-3' and 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC-3'. The mRNA from the RB1 gene was amplified using the previously described primers: C3-5, 5'-TACTGCAAATGCAGAGACACA-3' and C4-3, 5'-TGTTCCCTCCAGGAATCCGTA-3' (Mori, N., et al., *Oncogene* 5:

- 7 -

1713-1717 (1990)). Both of these primer pairs were chosen to span intron sequences in order to ensure that the resulting products were not due to amplification from genomic DNA. Brush-1 mRNA was amplified using primers homologous to regions within the Brush-1 sequence: 5'-TTAGTGGCACTTTATTC-3' and 5'-CATCAGTGTAGCCA AGC-3'. This primer pair does not span an intron and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was conducted both with and without the RT enzyme to assure that results did not reflect DNA contamination. The PCR reaction mixture (50 μ L final vol.) consisted of: template cDNA, 1.5 mM $MgCl_2$, 200 μ M dNTPs (each), 20 pmol of each primer pair and 1 U of Taq DNA polymerase (Promega). The entire PCR mixture was heated to 95°C for 3 min to assure complete dissociation of the template sequences and then subject to 35 cycles of amplification under the following conditions: 95°C for 30 s, 50°C for 30 s and 72°C for 3 min with a final extension at 72°C for 10 min. The PCR products were separated on a 1% agarose gel and ethidium bromide stained for visualization.

LOH analyses used DNA from both tumors and normal tissues. DNA aliquots (40 ng) were used as templates for PCR amplification of polymorphic markers (Weissenbach, J., et al., *Nature (Lond.)*, 359: 794-801 (1992)) using primers specific for the D13S219 region at 13q13 (Research Genetics, Huntsville, AL). These primers flank a CA repeat polymorphism localized to chromosome 13q13, proximal to RB1. Primers for the RB1 gene (Brandt, B., et al., *Am. J. Hum. Genet.* 51: 1450-1451 (1992) flank a variable number terminal repeat (VNTR) which is highly polymorphic. PCR conditions used for the LOH analyses at both the D13S219 and RB1 sites are those described for the RT-PCR analyses with the following

- 8 -

modifications: Cycle conditions for D13S219 were 35 cycles of amplification under the following conditions: 94°C for 30 s, 56°C for 30 s and 72°C for 30 s with a final extension at 72°C for 10 min.

5 Cycle conditions for RB1 VNTR were 35 cycles of amplification under the following conditions: 95°C for 45 s, 53°C for 25 s and 72°C for 2 min. with a final extension at 72°C for 10 min. All PCR products were separated by electrophoresis on a 6%
10 polyacrylamide gel and stained with ethidium bromide for visualization. PCR products from amplification of paired normal and tumor DNA from the same individual were compared to determine first, if the individual was heterozygous at the tested site and
15 second, whether one of the alleles had been lost indicating that there had been a loss of heterozygosity (LOH).

The 4.11N 1.5 kb cDNA fragment (see Figure 3) was subcloned into the Bluescript plasmid
20 (Stratagene). Both strands of the cDNA fragment were sequenced using the Sequenase 2.0 system of dideoxynucleotide chain termination (US Biochemicals). The sequence was analyzed using the Eugene (Baylor College of Medicine) sequence analysis
25 program. The Brush-1 cDNA fragment was labeled with ³²P-dCTP using the Multiprime labeling system (Amersham). This probe was used to screen a total of 5 X 10⁵ independent clones from an EMBL-3 human placental genomic library (Clontech, Palo Alto, CA).
30 Two genomic clones corresponding to this cDNA were isolated.

One additional cDNA clone, designated 4.11T, was isolated by using the 4.11N as a probe for screening a cDNA library derived from breast tumor
35 mRNA. Two other clones, designated 4.11K1 and 4.11K2 were isolated, and the relative positions of the

- 9 -

clones within the mRNA were determined using the RACE method as described by Frohman, et al., *Proc. Natl. Acad. Sci. USA* **85**: 8998-9002 (1988) (see Figure 3).

The Northern blots were prepared as described by Sambrook, J., Fritsch, E.F. & Maniatis, T., *MOLECULAR CLONING: A LABORATORY MANUAL* (1992) using 10 ug of poly-A⁺ selected RNA. These were probed using the radioactively labelled cDNA fragment described above. The EMBL-3 genomic DNA clones described above served as Brush-1 templates for the FISH analysis. The RB probe and methods used for the FISH analysis are previously described in Kallioniemi, A., et al., *Cytogenet. Cell Genet.* **60**: 190-193 (1992).

The Brush-1 mRNA was initially detected on Northern gels of normal breast epithelium RNA at levels comparable to those seen for RB1 (see Figure 1). Brush-1 codes for a single 4.7 kb mRNA. An RT-PCR approach was used for a survey of breast cancer cell lines in order to compare expression for Brush-1 with RB1 (which also codes for a 4.7 kb mRNA). An example of this is seen in Figure 2 where RNAs from normal breast epithelium and three breast cancer cell lines were analyzed with this RT-PCR technique. After the initial RT step, three different amplicon sets were used to analyze each of the newly synthesized cDNAs. The first amplicon set was specific to the Brush-1 mRNA, the second was specific to the RB1 mRNA and the third, for β -Actin, served as a control. The expected sizes of the amplified DNA products were 592, 539 and 1126 base pairs for Brush-1, RB1 and β -Actin, respectively. Two of the breast cancer cell lines (CAMA1 and DU4475) have negligible expression for Brush-1 whereas both the normal breast epithelium (G94) and another breast cancer cell line

- 10 -

(MDA468) express high levels of the Brush-1 mRNA. All four types of breast cells have high levels of β -Actin expression and only DU4475 cells do not express RB1.

5 This survey was extended to additional samples of both normal breast epithelium and breast cancer cell lines. The results are shown in Table 1.

Table 1

10	<u>Types of Specimen</u>	<u>Expression of Brush-1^a</u>	<u>Expression of RB1^a</u>	<u>LOH at 13q13- q14^b</u>
	A. Primary Breast Tumors			
	B200	+	+	-
	B201	+	+	-
15	B212	+	+	-
	B381	+	+	-
	B317	-	+	+
	B349	-	+	+
	B398	-	+	+
20	B406	-	+	+
	B. Cells in culture			
	1. Normal Mammary Epithelium			
25	337EA	+	+	
	998E	+	+	
	1130E	+	+	
	G61E	+	+	
	G94E	+	+	
30	2. Breast Cancer Cell Lines			
	BT20	+	+	
	MCF7	+	+	
	BT474	+	+	
35	MDA157	+	+	
	MDA231	+	+	
	MDA468	+	+	
	MPE600	+	+	
	CAMA1	-	+	
40	MDA435	-	+	

- 11 -

MDA134	-	+
SKBR3	-	+
UACC812	-	+
DU4475	+/-	-

- 5 a Detection of specific mRNAs by RT-PCR: (+) = presence; (-) = absence, (+/-) = greatly reduced levels.
- b Detection of LOH: (+) = LOH for the region; (-) = no LOH detected for the region.
- 10 All five normal breast epithelial cell cultures expressed high levels of Brush-1 mRNA. In contrast, 6 of 13 breast cancer cell lines produced greatly reduced levels of Brush-1 mRNA. It appears that the Brush-1 gene shows no expression in five of these
- 15 cell lines and only at very low levels for DU4475. These low mRNA levels were consistently observed by both RT-PCR and Northern analyses (data not shown). Conversely, RB1 mRNA is expressed in all normal and breast cancer cell lines except DU4475. The Brush-1
- 20 mRNA, therefore, shows much more differential expression in the cancer cell lines than RB1.

The Brush-1 tumor suppressor gene was detected in formalin fixed tissue section using in situ reverse transcriptase polymerase chain reaction

25 (in situ RT PCR). Brush-1 cDNA was synthesized in situ by reverse transcription using a Brush specific oligonucleotide primer. In situ polymerase chain reaction amplification in the presence of digoxigenin-11-dUTP and subsequent binding with an

30 antidigoxigenin antibody conjugated to alkaline phosphatase allowed direct visualization. Brush-1 is expressed in the luminal layer of epithelial cells of lobules and ductules in tissue sections from normal reduction mammoplasties (5 patients). In sections of

35 invasive carcinoma, the tumor suppressor gene is expressed in about 10% of the invasive tumor cells (7

patients). In cases of invasive carcinoma that contain a loss of heterozygosity (LOH) in the 13q13-14 region, no tumor cells express Brush-1. The specificity of the in situ reaction described above

5 was demonstrated by performing a reaction without reverse transcriptase and also eluting the amplified fragments from the sections and detection by agarose gel electrophoresis. These results show that in vivo the ~~Brush~~-1 gene has the expression pattern expected

10 for a tumor suppressor gene. For immunodetection of Brush-1 message in tissue from reduction mammoplasties, archival formalin/alcohol fixed, paraffin embedded sections were subjected to RT in situ PCR. Lobules and ducts from the same section

15 with no RT step and with the RT step were compared. The results showed that Brush-1 message is expressed at high levels in epithelial cells, and the sections with no RT step are negative.

Immunodetection of Brush-1 message in tumor

20 cells displayed a loss of heterozygosity in the 13q13-14q region, proximal to the retinoblastoma gene. Archival formalin fixed, paraffin embedded sections were subjected to RT in situ PCR and an area of invasive tumor cells was examined having a section

25 with RT step. The results show tumors bearing an LOH at 13q 13-14 do not express Brush-1 message.

Since in situ RT PCR is applicable to any in vivo system and is capable of detecting low copy mRNAs, it is useful in studying tumor suppressor gene

30 expression in vivo in rare and difficult to obtain cells. Therefore, molecules may be investigated for which there are no antibodies available, as is the case with Brush-1. Since the method is based on the incorporation of digoxigenin-11-dUTP during

35 amplification and then immunodetection, it is rapid,

- 13 -

requiring less than two days, and there is essentially no non-specific binding. Moreover, less than 20 copies of mRNA per cell can be detected. See Nuovo, GJ et al. Am J Pathol 1991, 189:847; Heniford, BW et al. NAR 1993, 21:3159. Therefore, this technique is well suited to the study of single cells obtained from nipple aspirates. It is also well suited for the study of heterogeneous cell populations where only a few cell express the gene of interest. Such a study was done on peripheral blood cell from patients with HIV where it was shown for the first time by in situ RT-PCR that some leukocytes harbor the HIV virus (Nuovo, GJ et al. J. of acquired Immune Deficiency Syndromes, 1994 7:916).

The Brush-1 cDNA probe is a 4.3 kb sequence assembled from Brush-1 cDNA clone fragments. The correct 5'-3' orientation for each fragment was determined by Northern hybridization of separate single-stranded riboprobes complementary to each of the cDNA strands. Only one orientation hybridized to the 4.7 kb mRNA. SEQ ID NO:1 is the sequence for this cDNA probe. Sequence analysis revealed no significant homology to any known sequences in the Genbank. A longer cDNA sequence (4.3 kb) which hybridizes to the mRNA is SEQ ID NO:2 assembled from fragments used to assemble SEQ ID NO:1 and additional clone fragments. SEQ ID NO:1 is contained within SEQ ID NO:2 and begins at position 818 in SEQ ID NO:2.

The relationship of Brush-1 to RB1 was first suggested by preliminary mapping of the gene to chromosome 13. The cytological position of Brush-1 relative to RB-1 was done by FISH analysis. Brush-1 is localized to a single position at 13q12-q13, proximal to RB1. Previous studies by Lundberg, C., et al., Proc. Natl. Acad. Sci. USA 84: 2372-2376

- 14 -

(1987) and Devilee, p., et al., *Genomics* 5: 554-560 (1989) found that many breast cancers showed LOH over a large region which included 13q12-13 and that in one primary breast tumor the LOH on chromosome 13q included 13q12-q13 but did not include RB1. Since Brush-1 is proximal to RB1, and Brush-1 is often lost in breast cancer cell lines, it is a useful diagnostic tool for identifying primary breast tumors.

10 The LOH at D13S219, located at 13q13, was surveyed for and compared to LOH found at the RBI gene. In one survey of 108 primary breast tumors it was found that LOH at the RBI gene was 45%. Another survey of 76 tumors from the same population gave 42%
15 LOH for D13S219. In all cases where the samples were informative for both RBI and D13S219, the results were identical for LOH. A selection of these tumors were examined for expression of the Brush-1 and RB1 mRNA (Table 1). Four tumors with no LOH in this
20 region demonstrated expression for both Brush-1 and RB1. In contrast, four tumors which clearly demonstrated LOH at both D13S219 and RB1, all showed decreased expression for Brush-1 while maintaining normal levels of expression for RB1. This
25 differential loss of Brush-1 expression, therefore, is manifest in both breast cancer cell lines and primary breast tumors.

 The Brush-1 mRNA is thus useful as a diagnostic marker for breast cancer. The Brush-1
30 cDNA probe, or substantially identical sequences, that is, sequences having 90% or greater homology with the Brush-1 cDNA probe, may be used to detect the presence of this marker in breast tissue or cell samples by generally applied molecular techniques
35 known to those of ordinary skill in the art, such as

- 15 -

Northern analysis and *in situ* hybridization. By comparison of the differential amounts of mRNA in the suspected tumor cells and normal cells, the likelihood of the presence of breast cancer can be ascertained. In addition, the probe may be used in the design and manufacture of new drugs for the treatment of breast cancer. The Brush-1 RNA (or DNA) may be used as gene therapy agents to provide missing tumor suppressor function where it is naturally lacking.

We also noted from a BLAST search that the 5' of Brush-1 from the start of the cDNA is 98% homologous (in anti-sense relationship) to the actin binding protein ABP-280 (8360bp; see J.Cell Biol. 111(3):1089-105(1990)). This suggests that Brush-1 could act as antisense RNA to block RNA translation for ABP-280 which is important to cell growth and motility.

In addition, lack of or abnormally low expression of a Brush-1 gene or the presence of a mutant Brush-1 gene in a patient may predispose that individual to breast or other types of cancer. Such a familial breast cancer gene, BRCA1, had been previously localized to chromosome 17 and the target gene has now been identified [Miki et al., 1994, Science 266:66-71; Futreal et al., 1994, Science 266:120-122]. Recently, a second familial breast cancer gene, BRCA2, was localized to chromosome 13q12-13 [Wooster et al., 1994, Science 265:2088-2090]. Furthermore, the BRCA2 gene was most closely linked to the polymorphic microsatellite repeat marker D13S260. Yeast Artificial Chromosomes (YACs) isolated with the D13S260 markers were tested for the presence of the Brush-1 gene. Of eight YACs tested, two were conclusively shown to contain the Brush-1

- 16 -

gene. Therefore, this second familial breast cancer gene is in the same chromosomal location (13q12-13) as Brush-1 and Brush-1 is contained in the chromosomal segment showing the strongest linkage to
5 BRCA2.

The nucleotide sequences of this invention used for diagnostic applications ~~may be the~~ entire sequence of the gene or may be ~~fragments thereof~~ based on restriction enzyme ~~digestion~~ (which fragments may be
10 all or part of the open reading frames) untranslated regions, intermediate coding regions, and fragments and combinations thereof. The minimum size single-stranded fragment will be at least 20 bases and usually at least 50 bases and may be 100 bases or
15 more. The sequence may be obtained as a fragment or be synthesized.

The fragments can be used in a wide variety of ways, depending upon their size, their natural function, the use for which they are desired, and the
20 degree to which they can be manipulated to modify their function. Thus, sequences of at least 20 bases, more usually at least 50 bases, and usually not exceeding about 1000 bases, more usually not exceeding about 500 bases, may serve as probes for
25 detection of the presence of *Brush-1* in a host tissue, including the genome, or in a physiological fluid, such as blood, lymph, saliva, spinal fluid, or the like. These sequences may include coding and/or non-coding sequences.

30 Where the nucleotide sequences are used for duplex formation, hybridization, or annealing, for example, for diagnosis or monitoring of the presence of the *Brush-1* in vivo or in vitro, complete base pairing will not be required. One or more mismatches

- 17 -

are permissible. To ensure that the presence of one or a few, usually not more than three, mismatches still allows for stable duplexes under the predetermined stringency of hybridizing or annealing conditions, probes will normally be greater than 20 bases, preferably at least about 50 bases or more.

The method of detection will involve duplex formation by annealing or hybridization of an oligonucleotide probe, either labeled or unlabeled, depending upon the nature detection system, with the DNA or RNA of host tissue suspected of harboring *Brush-1*. A physiological sample may include tissue, blood, serum, etc. Particularly, blood samples will be taken, more particularly blood samples containing peripheral mononuclear cells, which may be lysed and the DNA or RNA isolated in accordance with known techniques.

The sample polynucleotide mixture obtained from the human host can be bound to a support or may be used in solution depending upon the nature of the protocol. The well-established Southern technique [(1975) J. Mol. Biol. 98:503] may be employed with denatured DNA, by binding the single-stranded fragments to a nitrocellulose filter. Alternatively, RNA can be blotted on nitrocellulose following the procedure described by Thomas, (1980) Proc. Natl. Acad. Sci. (USA) 77:5201. Desirably, the fragments will be electrophoresed prior to binding to a support, so as to be able to select for various sized fractions. Other techniques may also be used such as described in Meinkoth & Wahl, (1984) Anal. Biochem. 138:267-284.

The oligonucleotide probe may be DNA or RNA, usually DNA. The oligonucleotide sequence may

-18-

be prepared synthetically or in vivo by cloning, where the complementary sequence may then be excised from the cloning vehicle or retained with the cloning vehicle. Various cloning vehicles are available, such as pBR322, M13, Charon 4A, or the like, desirably a single-stranded vehicle, such as M13.

As indicated, the oligonucleotide probe may be labeled or unlabeled. A wide variety of techniques exist for labeling DNA and RNA. As illustrative of such techniques, is radiolabeling using nick translation, tailing with terminal deoxytransferase, or the like, where the bases which are employed carry radioactive 32 P. Alternatively, radioactive nucleotides can be employed where carbon, nitrogen or other radioactive atoms may be part of the nucleoside structure. Other labels which may be used include fluorophores, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, or the like. Alternatively, instead of having a label which provides for a detectable signal by itself or in conjunction with other reactive agents, ligands can be used to which receptors bind, where the receptors are labeled such as with the above-indicated labels, which labels provide detectable signals by themselves or in conjunction with other reagents See, e.g., Leary et al. (1983) Proc Natl. Acad. Sci. (USA) 80:4045-4049; Cosstick et al. (1984) Nucleic Acids Res. 12:1791-1810.

The oligonucleotide probes are hybridized with the denatured human host nucleic acid, substantially intact or fragmented, or fractions thereof, under conditions of predetermined stringency. The stringency will depend upon the size and composition of the probe, the degree of mismatching, and the like. Usually, an organic

-19-

solvent such as formamide will be present in from about 30 to 60 vol percent, more usually from about 40 to 50 vol percent, with salt concentration from 0.5 to 1M. Temperatures will generally range from about 300 C to 650 C., more usually from about 350 C. to 500 C. The times for duplex formation may be varied widely, although minimum times will usually be at least about one hour and not more than about 72 hours, the time being selected in accordance with the amount of DNA or RNA available, the proportion of DNA or RNA as compared to total DNA or RNA, or the like. Stringency may also be modified by ionic strength and temperature. The hybridization and annealing can be carried out in two stages: a first stage in a hybridization medium; and, a second stage, involving washings at a higher stringency, by varying either or both temperature and ionic strength.

As understood in the art, the term "stringent hybridization conditions" as used herein refers to hybridization conditions which allow for closely related nucleic acid sequences to duplex (e.g., greater than about 90% homology), but not unrelated sequences. The appropriate conditions can be established by routine procedures, such as running Southern hybridization at increasing stringency until only related species are resolved and the background and/or control hybridization has disappeared (i.e., selective hybridization).

Nucleotide probes may be prepared employing reverse transcriptase using primers, e.g., random primers or specific primers. The cDNA may be prepared employing a radioactive label, e.g., 32 P, present with one or more of the dNTPs. Reverse transcription will provide various sized fragments depending on the primers, the efficiency of

-20-

transcription, the integrity of the RNA, and the like. The resulting cDNA sequences may be cloned, separated and used for detection of the presence of *Brush-1* in the human genome.

5 Using specific primers of 10 to 20 bases, or more, *Brush-1* may be reverse transcribed and the resulting ss DNA used as a probe specific for the region which hybridized to the primer. By employing one or more radionucleotide-labeled bases, the probes
10 will be radiolabeled to provide a detectable signal. Alternatively, modified bases may be employed which will be randomly incorporated into the probe and may be used to provide for a detectable signal. For example, biotin-modified bases may be employed. The
15 resulting biotin-containing probe may then be used in conjunction with labeled avidin to provide for a detectable signal upon hybridization and duplex formation.

The *Brush-1* sequence may also be used therapeutically
20 through gene therapy on subjects identified as having a genetically aberrant *Brush-1* gene. The subjects will then have normal *Brush-1* DNA and the ability to utilize its tumor-suppressing activity. For example *Brush-1* DNA may be injected into subject after being
25 based to an appropriate vector, such as viral vectors, liposomes and other vectors known in the art.

-21-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: SCHOTT, D.

(ii) TITLE OF INVENTION: CDNA PROBE FOR BREAST
5 CANCER DIAGNOSIS
AND TREATMENT

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

10 (A) ADDRESSEE: REGINALD J. SUYAT, ESQ.
(B) STREET: 333 BUSH STREET
(C) CITY: SAN FRANCISCO
(D) STATE: CALIFORNIA
(E) COUNTRY: USA
15 (F) ZIP: 94104-2878

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
20 (D) SOFTWARE: PatentIn Release #1.0,
Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US
(B) FILING DATE:
25 (C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/160,088
(B) FILING DATE: 30-NOV-1993

(vii) PRIOR APPLICATION DATA:

30 (A) APPLICATION NUMBER: US 08/314,598
(B) FILING DATE: 27-SEP-1994

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: SUYAT, REGINALD J.
(B) REGISTRATION NUMBER: 28,172
35 (C) REFERENCE/DOCKET NUMBER: 11561-0026

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 415-772-6432
(B) TELEFAX: 415-772-6268

(2) INFORMATION FOR SEQ ID NO:1:

40 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3542 base pairs

-22-

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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AACAGCATCT GCTTTCCTCT      720

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35 CCGCTTGGT TTTTCCTTTT      900
```


-23-

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5 TGCTCTGCTG AATAATTTCA TTACCTCTGC ACATGCCTGT
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 CACCGCTGAG CTGGGCAGAG 1320

15 CTGCGGCACA GGGCCTGGGC TGCAAAGGTG CCCTGCTCCT
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 20 TTAGGTGAAA GTAGATATTG 1500

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25 AAGCACCTCA TGAGATTCCC TTGATCAGCC CTGCAGCTGT
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 30 ACTGTGTTGA ACAAACAGGT 1800

GCTCCAGGCT TTGATTATAG ATACGACTTC AAAAATATGC
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GGACTTTAAC CTA CTCAACA GTATTTTCATA TCCATTGTGG
 TTAGT TACTC AGTTATGTTG 1920

35 AGAAGAATCT GGAGCTAAAA GCAGAGATGT TTGAGGTGAC
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-24-

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CAGTGATTGT ACAGAGCCTG 2100

5 TCCATTGGGT GCAGTCATGT AGATCTGAAG CCCTGAAAAG
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GCTATTCTTA GGACTTCTGC 2400

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20 GGAGAGAACA CAGAAGCTAC TATCCATGTC AGGATTTATT
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GACTTGCGAA AAAGCTTTTG 2700

25 CCCTGTTGTG TACCATAACA TTTAAAAGAA TGGAAAATGA
CTGAAATCCA ATTTAGATTA 2760

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-25-

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AGCTTGGCTA CACTGATGTT 3180

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TTGCAGTATT ACCAATGCTG 3420

TTGTAAATTG AATTTAAAGT GGTATTAAAA AAAACTGTTA
AACAATTTTT ATCTGTTTGT 3480

15 ATATCTTACT ATAGATTATG TACAAGTAAC ATCTAAATAA
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AA
3542

(2) INFORMATION FOR SEQ ID NO:2:

- 20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4359 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- 25 (ii) MOLECULE TYPE: cDNA to mRNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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CATTCACCTCT CAAATGGTCC AGAGAAAAGT GTCTGTGTCT
GTCTGTGTAA AGATGATAGG 180

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GGGAATCCAA GTATATGGGA 240

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AATTATTTCA AAACAGTTAA 300

-26-

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	GCACTCCAGC	CTGGGTCACA	GAGTGAGACC	CAATCTCAAA
	AAAAAAAAAA	ATGGATAAAC	540	
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	GCATAGCACC	TTTTGTATAA	1380	

-27-

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-28-

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25 GGGCCTACTA CATTGGCGCT ATTCTTAGGA CTTCTGCAAC
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-29-

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10 ATAAATTGTA AGGTAATTTT AAATTGTCCC TCGTATTATT
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AAAATGTTTT CTGAGCTCCA 4080

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25 ATTAAAAAAA ACTGTAAAC AATTTTATC TGTTTGTATA
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4359

-30-

WHAT IS CLAIMED IS:

1. Isolated DNA comprising a nucleotide sequence substantially identical to SEQ ID NO:1.
2. Isolated DNA comprising a nucleotide sequence
5 substantially identical to SEQ ID NO:2.
3. A diagnostic agent for detecting tumors and pre-malignant cells in host tissue or cells comprising at least one probe selected from the group consisting of a DNA sequence substantially identical to SEQ ID
10 NO:2, its complementary DNA sequence, an RNA sequence substantially identical to the RNA complement of SEQ ID NO:2, the complement of said RNA complement, and fragments of said sequences; and a marker on said probe.
- 15 4. A pharmaceutical composition for providing or enhancing the tumor-suppressing ability of a subject comprising a therapeutically effective amount of mRNA, cDNA of said mRNA or DNA sequences of genomic DNA of BRUSH-1, and a pharmaceutically acceptable
20 carrier.
5. A diagnostic agent for detecting tumors and pre-malignant cells in host tissue or cells comprising at least one probe selected from the group consisting of a DNA sequence substantially identical to SEQ ID
25 NO:1, its complementary DNA sequence, an RNA sequence substantially identical to the RNA complement of SEQ ID NO:1, the complement of said RNA complement, and fragments of said sequences, and a marker on said probe.

-31-

6. A diagnostic method for detecting the presence of breast tumors or pre-malignant breast cells in a subject comprising the steps of
- (a) contacting a library containing mRNA
5 derived from tissue or cells from said subject with one or more labelled probes, said probes comprising DNA segments of SEQ NO:1 or SEQ NO:2;
 - (b) separating the probes which hybridize with mRNA from said library;
 - 10 (c) identifying the mRNA sequences from step (b) and determining if said sequences define a normal amount of normal BRUSH-1 gene in said subject.
7. A therapeutic method for treating tumors or pre-malignant cells in a subject comprising the steps of
- 15 introducing into a subject known or suspected of having tumors, an agent comprising RNA or DNA or selected from the group consisting of mRNA, cDNA and genomic DNA of BRUSH-1, which enables said subject or augments the ability of said subject to suppress
- 20 tumor proliferation or growth.
8. A method of providing a subject deficient in a functional BRUSH-1 gene with a functional BRUSH-1 gene, comprising the step of introducing into said subject an agent comprising RNA or DNA selected from
- 25 the group consisting of mRNA, cDNA and genomic DNA of BRUSH-1, which enables said subject or augment the ability of said subject to suppress tumor proliferation or growth.
9. A method according to either Claim 7 or 8
- 30 wherein said agent comprises a substantially similar variant of said RNA or DNA.
10. A method according to Claim 6 where said probes are labelled.

1/3

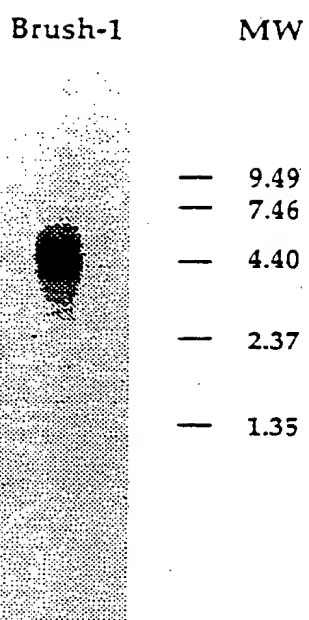


FIGURE 1

2 / 3

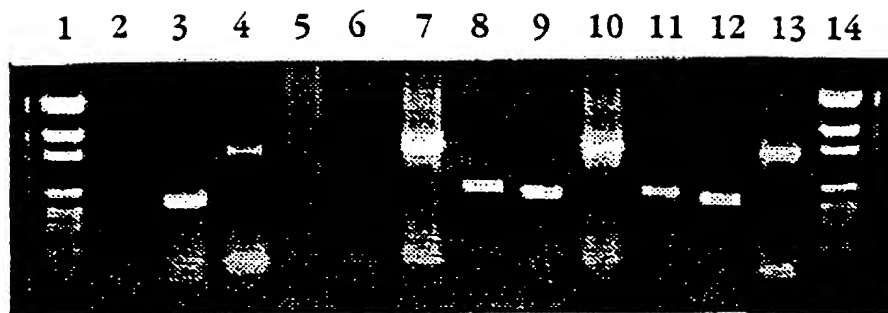
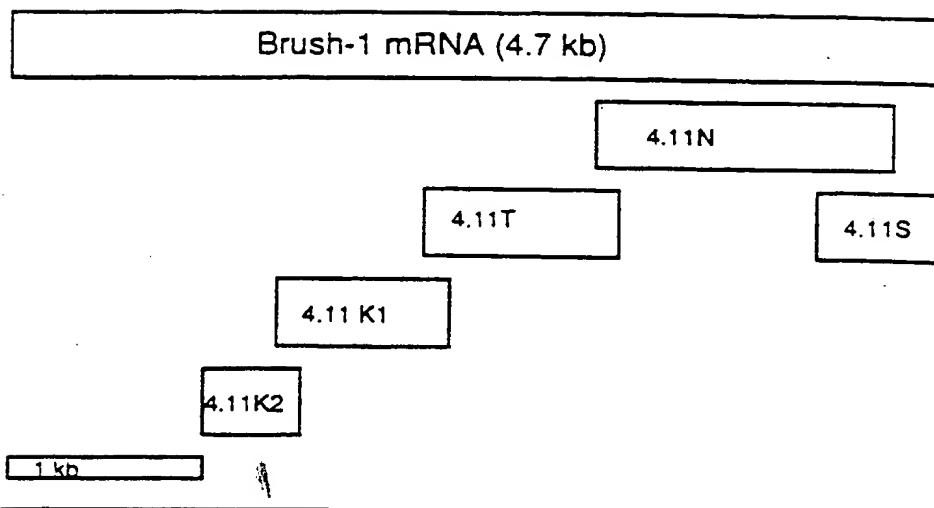


FIGURE 2

3 / 3

**FIGURE 3**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/13823

A. CLASSIFICATION OF SUBJECT MATTER IPC(6) : C07H 21/02, 21/04; C12Q 1/68; A61K 48/00 US CL : 435/6; 536/24.31; 514/44 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/6; 536/24.31; 514/44 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Please See Extra Sheet.		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. SAMBROOK et al, "MOLECULAR CLONING, A LABORATORY MANUAL" published 1989 by Cold Spring Harbor Laboratory Press (Plainview, New York), pages 7.49-7.52, 7.54, 7.55, and 9.52-9.57, see pages 7.49-7.52, 7.54, 7.55, and 9.52-9.57.	3, 5, 6, 10
Y	The Lancet, Volume 339, issued 21 March 1992, A. A. Gutierrez et al, "Gene Therapy for Cancer", pages 715-721, especially Table IV.	4, 7-9
X,P ----- Y,P	Cancer Research, Volume 54, issued 15 March 1994, D. R. Schott et al, "A Candidate Tumor Suppressor Gene in Human Breast Cancers", pages 1393-1396, especially Table 1 and Figures 2 and 3.	1-3, 5, 6, 10 ----- 4, 7-9
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* "A" "E" "L" "O" "P"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance earlier document published on or after the international filing date document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family
Date of the actual completion of the international search 23 FEBRUARY 1995		Date of mailing of the international search report 13 MAR 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer <i>Nathaniel Feja for</i> DAVID SCHREIBER Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/13823

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	Nature Genetics, Volume 4, issued August 1993, M. D. Adams et al, "Rapid cDNA sequencing (expressed sequence tags) from a directionally cloned human infant brain cDNA library", pages 373-380.	1, 2 ----- 3, 5, 6, 10
X,P ----- Y,P	GenBank, Locus HHEA05L, 06 May 1994.	1, 2 ----- 3-10
X,P ----- Y,P	GenBank Locus HSC1IC112, 04 November 1994.	1,2 ----- 3-10
X,P ----- Y,P	GenBank Locus S69790, 22 September 1994.	1,2 ----- 3-10
X ----- Y	GenBank Locus T08945, 03 August 1993.	1,2 ----- 3-10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/13823

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
~~because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:~~

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐
☐

- The additional search fees were accompanied by the applicant's protest.
No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/13823

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

GENBANK, GENBANK-NEW, UEMBL, EMBL-NEW, N-GENESEQ, MEDLINE, CA, APS, BIOSIS, WPI
search terms: gene replacement therapy, hybridization, BRUSH-1, breast cancer, LOH, chromosome 13, DNA, RNA

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-6 and 10, drawn to an isolated DNA comprising a nucleotide sequence substantially identical to SEQ ID NO:1 or 2; a diagnostic agent for detecting tumors and pre-malignant cells using nucleic acid having the sequence SEQ ID NO:1 or 2 or its complement or fragments of said sequences, a pharmaceutical composition comprising a therapeutically effective amount of BRUSH-1 mRNA, cDNA, or genomic DNA, and a method of diagnosis for detecting breast tumors or pre-malignant cells by determining the quantity of BRUSH-1 mRNA.

Group II, claims 7-9, drawn to a method for introducing into a subject an agent comprising mRNA, cDNA or genomic DNA of BRUSH-1 or a similar variant, which enables said subject or augments the ability of said subject to suppress tumor proliferation or growth.

PCT Rule 13.1 recites the basic principle of unity of invention that an application should relate to only one invention or, if there is more than one invention, that applicant would have a right to include in a single application only those inventions which are so linked as to form a single general inventive concept. According to PCT Rule 13.1, a group of inventions is linked to form a single inventive concept where there is a technical relationship among the inventions that involves at least one common or corresponding special technical feature that defines the contribution which each claimed invention, considered as a whole, makes over the prior art.

The two inventions of this application consist of: Group I, isolated DNA or RNA and a method of using such DNA or RNA for detecting the presence of breast tumors and Group II, a method for introducing into an subject, an agent comprising BRUSH-1 RNA or DNA, which enables the subject or augments the ability of the subject to suppress tumor proliferation and growth. The two inventions are not linked by a special technical feature within the meaning of PCT Rule 13 for the following reasons: The two groups are not linked to the BRUSH-1 DNA by a special technical feature because the methods of the claims share a technical relationship that involves a corresponding special technical feature that does not define the contribution which each claimed invention, considered as a whole, makes over the prior art since detection of a disease by hybridization and treatment of a disorder by gene therapy are well known in the art. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.